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Sustained Expression of Therapeutic Level of Factor IX in Hemophilia B Dogs by AAV-Mediated Gene Therapy in Liver

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We demonstrate that a single intraportal vein injection of a recombinant adeno-associated virus (rAAV) vector encoding canine factor IX (cFIX) cDNA under the control of a liver-specific enhancer/promoter leads to a long-term correction of the bleeding disorder in hemophilia B dogs. Stable expression of the therapeutic level of cFIX (5% of normal level) was detected in the plasma of a dog injected with an AAV vector at a dose of 4.6×10^{12} particles/kg for over 7 months. Both whole-blood clotting time (WBCT) and activated partial thromboplastin time (aPTT) of the treated dogs have been greatly decreased since the treatment. No anti-canine factor IX antibodies have been detected in the treated animals. Importantly, no bleeding has been observed in the dog that expresses a therapeutic level of cFIX for 7 months following vector administration. Moreover, no persistent significant hepatic enzyme abnormalities were detected in the treated dogs. Thus, a single intraportal injection of a rAAV vector expressing cFIX successfully corrected the bleeding disorder of hemophilia B dogs, supporting the feasibility of using AAV-based vectors for liver-targeted gene therapy of genetic diseases.

Key Words: **AAV vectors; hemophilia B; factor IX; intraportal; expression; hemophilic dogs; liver enzyme.**

INTRODUCTION

Hemophilia B is an X-linked bleeding disorder caused by a defect or deficiency in blood coagulation factor IX. Current treatment for hemophilia B consists of intravenous infusion of clotting factor IX (FIX) concentrates, either plasma derived or recombinant. Because of the safety and expense of replacement products, infusions are generally given only in response to bleeds and not prophylactically. Gene therapy could provide sustained expression of functional clotting factor in the circulation; thus, bleeds could likely be prevented rather than treated on demand. A major goal of gene therapy for hemophilia B has been to achieve long-term expression of therapeutic levels of FIX (1, 2) without generating any toxic side effects.

Recombinant adeno-associated viral (rAAV) vectors appear to be one of the most promising vectors for *in vivo* gene therapy of hemophilia B (3, 4). A number of studies have reported long-term and high level expression of FIX in immunocompetent mice following a single administration of rAAV vectors (5–8). Additionally, phenotypic correction of the bleeding disorder has been achieved in factor IX knockout mice (7, 8). Recently, several groups have scaled up 25- to 50-fold to apply similar strategies to hemophilia B dogs and achieved sustained expression of cFIX (7, 9, 10). However, so far the cFIX expression levels were not high enough to completely prevent the spontaneous and traumatic bleeding episodes in those treated dogs (7, 9–11).

We previously generated an AAV vector encoding cFIX driven by a strong liver-specific enhancer/promoter and demonstrated the long-term expression of above normal levels of cFIX and the complete cure of the bleeding disorder in immunocompetent hemophilia B mice following a single intraportal injection of the rAAV vector (8). In this study, we applied the similar strategy to a canine model of hemophilia B and achieved both sustained expression of the therapeutic level of cFIX and phenotypic improvement, as evidenced by a marked correction of the hemophilic coagulopathy and a reduction in bleeding episodes.

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MATERIALS AND METHODS

rAAV Vector Construction

AAV-LSP-cFIX was described previously (8). The vector plasmid pAAV-LSP-cFIX-WPRE was derived from plasmid pAAV-LSP-cFIX. It was constructed by inserting a 626-bp *EcoRV–XhoI* (blunt ended) fragment containing the woodchuck hepatitis posttranscriptional element (WPRE) from pBluescript KS(+)/WPRE-B11 (15) (a gift from Dr. Tom Hope) to the *PmeI* site (upstream of the poly(A)) of pAV-LSP-cFIX.

AAV vectors were made by three plasmid cotransfection methods as described by Xiao *et al*, (12). Vectors were recovered from two sequential CsCl gradients and dialyzed against sterile PBS. The physical vector titer was determined by a quantitative dot-blot assay (13). Titers were routinely in the range of $1–5 \times 10^{12}$ genomes/ml.

Intraportal Administration of rAAV Vectors

Hemophilia B dogs used in this study were produced at the Francis Owen Blood Research Laboratory at the University of North Carolina, Chapel Hill. All animals were treated according to the standards set in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23). All procedures were in accordance with institutional guidelines under approved protocols at the University of North Carolina. All dogs were placed under isoflurane (2–5%) anesthesia. Under sterile conditions, a midline laparotomy was performed. A balloon-tipped catheter was advanced into the portal vein under direct vision. The rAAV-cFIX vector was then infused directly into the portal vein with the inflated balloon. The infusion generally took from 30 to 60 min after which the catheter was removed. Canine plasma was used as a source of FIX before and briefly after surgery to prevent hemorrhage.

Coagulation Assays

The whole-blood clotting time (WBCT) and the activated partial thromboplastin time (aPTT) were performed as described (1, 2).

Canine Liver Enzymes

Canine blood chemistries (GGT, SGOT, SGPT, alkaline phosphatase, and bilirubin) were analyzed in an automated clinical laboratory.

Assays for Antigen Levels and Biological Activity of Canine FIX

Canine FIX antigen in dog plasma was determined by ELISA as previously described (14). Dog samples were diluted 1:20 to 1:100 depending on the cFIX expression levels in a given hemophilic dog. Pooled normal canine plasma (Sigma) was used as a factor IX standard (assumed to be 5 μg/ml). Clotting activity of cFIX in the treated dogs was determined by WBCT and aPTT assays as described (9).

Antibody Analysis

Antibodies against canine FIX were analyzed by ELISA as described previously (9).

RESULTS

Intraportal Delivery of rAAV to Hemophilia B Dogs

Previously, we constructed an AAV-LSP-cFIX vector in which the cFIX cDNA was under the control of a synthetic liver-specific enhancer/promoter, and a single intraportal injection of this vector into immunocompetent hemophilia B mice fully cured the bleeding disorder of hemophilia B mice (8). Recently, several reports have shown that the WPRE can enhance transgene expression in the context of HIV-, MLV-, and AAV-based vectors (15–17). To test if WPRE can enhance cFIX expression *in vivo*, we constructed AAV-LSP-cFIX-WPRE in which the WPRE was inserted between the 3′ end of the coding sequence of cFIX and poly(A) (Fig. 1). When tested in mice at comparable doses, AAV-LSP-cFIX-WPRE gave two- to threefold higher expression of cFIX than AAV-LSP-cFIX (L. Wang, unpublished data).

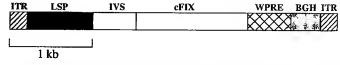

FIG. 1. Schematic drawing of the rAAV-cFIX-WPRE vector. The synthetic promoter (LSP) has been described before (8). Details of WPRE fragments used have been described (15).

The Chapel Hill hemophilia B dogs were used to test if cFIX rAAV can correct the bleeding disorder in a large animal model of hemophilia B. The molecular defect in these dogs is a missense mutation in the catalytic domain of factor IX that results in a complete lack of antigen in the plasma (18). Dogs selected for this study tested negative for anti-AAV neutralizing antibodies prior to treatment. Two hemophilia B dogs, C51 (female, 13.6 kg) and C52 (male, 17.6 kg), both 6.5 months old, were injected with 5×10^{12} particles of rAAV-LSP-cFIX via the portal vein. This dose is about 30-fold lower than what has been used for hemophilia B mice (in C57Blk/6 background) to achieve a 70% normal level of cFIX (8). Two additional dogs, C55 (male, 19 kg, 12 months old) and D39 (female, 5 kg, 2.8 months old), were injected with 8.7×10^{13} and 1.4×10^{13} particles of rAAV-LSP-cFIX-WPRE, respectively (Table 2).

Liver Enzymes in Transduced Dogs

Since the vectors were administered by direct intraportal vein injection, serum liver enzymes, bilirubin, and alkaline phosphatase concentration were assayed frequently during the first 2 weeks following treatment to monitor for any toxicity (Table 1). In general, no significant changes were detected except in one dog (C51) that had transient elevation of SGPT, one of the five indices of hepatic function tested. This dog had a baseline SGPT that was elevated, rose about four times the baseline on the second day following injection, and had returned to normal levels by day 8. This rAAV vector-induced hepatotoxicity is probably not due to the vector, since C52 received the same amount of vector from the same batch and did not show any abnormality in any of the liver enzyme assays. It is possible that another independent process was going on in this dog that was asymptomatic clinically since the SGPT was elevated on the day prior to treatment. Whatever the cause, it was minor and self-limited. All dogs had slightly elevated alkaline phosphatase activity, most likely due to bone growth in these young dogs, similar to what has been seen in other gene therapy studies that have utilized rAAV (7).

TABLE 1

Liver Enzyme Levels in rAAV-Transduced Hemophilia B Dogs

Dog	Day	SGOT[a]	SGPT[b]	Total bilirubin[c]	GGTP[d]	Alk Phos[e]
C51	0	66	128	0.2	4	162
	2	66	540	0.1	3	207
	3	44	368	0.1	4	213
	4	38	254	0.1	5	192
	8	38	105	0.2	4	177
	15	35	71	0.1	4	161
C52	0	32	43	0.2	3	154
	2	26	65	0.2	4	172
	3	28	63	0.1	4	175
	4	27	52	0.1	4	171
	8	37	40	0.2	4	161
	15	43	46	0.1	3	162
C55	0	31	36	0.1	2	80
	2	27	33	0.1	6	198
	3	21	12	0.1	9	150
	4	31	10	0.1	12	135
	8	42	12	0.1	9	101
	15	35	71	0.1	4	161
D39	0	18	17	0.3	1	24
	1	11	56	0.1	2	190
	2	12	51	0.1	4	199
	4	16	32	0.3	1	225
	7	24	29	0.3	2	239
	14	20	26	0.1	2	209
	21	19	23	0.1	2	171
	42	21	28	0.1	3	161
Normal range		15–66	12–118	0.1–0.3	1–12	5–131

[a] SGOT, serum glutamate–oxalacetate transaminase levels in IU/L.
[b] SGPT, serum glutamate–pyruvate transaminase levels in IU/L.
[c] Total bilirubin levels in mg/dL.
[d] GGTP, gamma glutamyltransferase in IU/L.
[e] Alk Phos, alkaline phosphatase levels in IU/L.

Sustained Expression of Biological Active cFIX in Hemophilia B Dogs

Following vector treatment, WBCT, aPTT, cFIX antigen in plasma, and cFIX antibodies in serum were monitored. The effects of the infused normal canine plasma on the WBCT, aPTT, and cFIX level diminish completely 3 weeks after the infusion (T. Nichols, unpublished data). Therefore, it should not interfere with the data that are 3 weeks after infusion. The kinetics of WBCT, aPTT, and the amount of cFIX detected in the serum is shown in Fig. 2. Table 2 summarizes the data and we conclude that all four dogs showed persistent shortening of WBCT, from >60 min (before treatment) to the average of 13.5–15.4 min for the first two hemophilic dogs (C51 and C52) treated with a lower dose of AAV-LSP-cFIX for over 1 year following injection and to the average of 9.9–9.1 min for the two dogs (C55 and D39) treated with a higher dose of AAV-LSP-cFIX-WPRE (Figs. 2a and 2b). While WBCT is sensitive to extremely low levels of FIX, aPTT is a much more frequently used and therefore clinically a more relevant parameter to monitor clotting activity. The aPTT value for hemophilia B dogs before treatment was 98–145 s and for normal dogs was 24–32 s. The first two dogs treated with a lower dose exhibited some shortening of the aPTT, to averages of 82 and 93 s, respectively (Fig. 2c). However, the two dogs injected with a higher dose showed dramatic shortening of aPTT, to averages of 51 and 62 s, respectively (Fig. 2d).

The secreted cFIX was detected on averages of 3.6 and 2.9 ng/ml in the plasma of the first two treated dogs (C51 and C52); however, this level of low expression has been sustained for over 1 year (Fig. 2e). In dog D39, which received AAV-LSP-cFIX-WPRE at a dose of 2.8×10^{12} particles/kg, the cFIX antigen level has been maintained at ~32 ng/ml for over 3 months, 10-fold higher than those of the first two dogs (Fig. 2e). The highest expression level of cFIX was achieved in dog C55, which received AAV-LSP-cFIX-WPRE at a dose of 4.6×10^{12} particles/kg. Three weeks posttreatment, the cFIX in plasma rose to 117 ng/ml, the cFIX expression level kept rising, and at week 7 it reached 218 ng/ml and remains unchanged for over half a year (Fig. 2f). The level of cFIX in

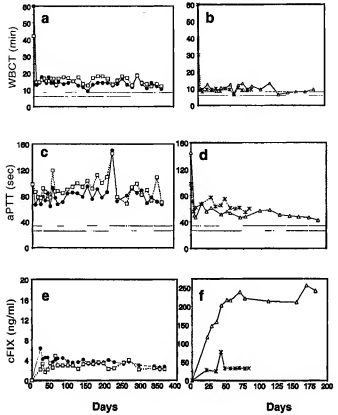

FIG. 2. Long-term expression of functional cFIX in hemophilia B dogs. Recombinant AAV-LSP-cFIX was administrated as a single intraportal injection into the liver of two hemophilia B dogs at a dose of 5×10^{12} particles (C51, ●; C52, □), and recombinant AAV-LSP-cFIX-WPRE was injected intraportally into two hemophilia B dogs in a dose of 8.7×10^{13} (C55, △) and 1.4×10^{13} particles (D39, *; vector introduced in D39 was stored at 4°C for 3 months). (a and b) WBCT; normal WBCT is 6–8 min. (c and d) aPTT; normal aPTT is 24–32 s. (e and f) Plasma cFIX antigen levels; therapeutic level is 250 ng/ml.

plasma of the C55 dog represents ~5% of the normal FIX plasma level (~5 μg/ml), a potentially therapeutic level for a hemophilia B patient. To date, no changes in the level of expression have been detected. For all four dogs, there were no antibodies detected against cFIX by ELISA (data not shown).

The Chapel Hill colony of hemophilia B dogs have an average of six bleeds per year that require treatment with normal canine plasma (T. C. Nichols, unpublished data). For our treated dogs, C51 had no bleeds post gene therapy, while C52 had two bleeding episodes during the 1-year period postinjection. D39 had five bleeds during the 180 days following vector transduction. C55, the one dog expressing the highest level of cFIX, had six bleeding episodes during the 1-year period before treatment; however, it has not had any bleeds for over 7 months following vector administration (Table 2). These data suggest that the phenotypic improvement and/or correction of bleeding disorder in hemophilia B dogs was due to the rAAV-mediated liver-targeted gene therapy.

Discussion

Recently AAV vectors have shown great promise as gene delivery vehicles for treatment of hemophilia B as evident from stable gene transfer of FIX in muscle and liver of factor IX-deficient murine and canine models (5–11, 19). Although muscle is readily accessible, liver is the natural site of FIX synthesis and may therefore be a more suitable target organ for efficient rAAV-mediated FIX expression. Besides, studies in immune-competent mice using rAAV-mediated muscle-targeted gene transfer did not generate detectable FIX in the plasma due to the formation of neutralizing antibodies against hFIX (19), and also transient antibodies against canine FIX were observed in one of the dogs after intramuscular injection of the AAV vector (9). In contrast, following intraportal administration of rAAV vectors, sustained expression of FIX (cFIX and hFIX) in plasma has been achieved in both immunocompetent mice and hemophilia B dogs (5, 7, 8), and antibodies against FIX were not detected by ELISA in our previous mouse study and in this study. Thus, it appears

TABLE 2

Hemophilia B Dogs Injected with rAAV Vectors Intraportally

Dog	Weight at injection (kg)	Age at injection (month)	Sex	Vector	Vector dose (particles/kg)	Total particles injected	Average WBCT[a] (min)	Average aPTT[b] (min)	Average cFIX in plasma[c] (ng/ml)	Bleeds post treatment
C51	13.6	6.5	F	AAV-LSP-cFIX	3.7×10^{11}	5×10^{12}	13.5 ± 2	81.9 ± 16.4	3.6 ± 0.5	0
C52	17.6	6.5	M	AAV-LSP-cFIX	2.8×10^{11}	5×10^{12}	15.4 ± 2.1	92.7 ± 17	2.9 ± 0.7	2
C55	19	12	M	AAV-LSP-cFIX-WPRE	4.6×10^{12}	8.7×10^{13}	9.9 ± 2.2	50.6 ± 4.7	218.1 ± 26.5	0
D39	5	2.8	F	AAV-LSP-cFIX-WPRE	2.8×10^{12}	1.4×10^{13}	9.1 ± 1.2	62.4 ± 5.8	31.7 ± 3.2	1[d]

[a] WBCT were >60 min (except C52 = 42 min) before injection. Average WBCT values after injection are given for dogs C51 and C52 (days 36–365), C55 (days 36–182), and D39 (days 35–83).

[b] Baseline aPTT (C51 = 98.4 s, C52 = 97.7 s, C55 = 145.1 s, D39 = 97.8 s). Average aPTT values after injection are given for dogs C51 and C52 (days 36–365), C55 (days 43–182), and D39 (days 35–83).

[c] Average cFIX values after injection are given for dogs C51 and C52 (days 36–365), C55 (days 36–182), and D39 (day 35 and days 48–83).

[d] In the last month there were five bleeds in this dog; however, we have not checked the WBCT, aPTT, and factor IX levels since 10/29/99.

that rAAV-mediated FIX expressed from the liver may be less immunogenic than that expressed from the muscle. It is formally possible that unlike adenoviral vectors, rAAV vectors are less efficient in transducing antigen-presenting cells and hence are less immunogenic.

Previously, we have demonstrated that a single intraportal injection of 2×10^{11} particles of AAV-LSP-cFIX gave rise to long-term expression of 70% of the normal level of functional cFIX in hemophilia B mice in a C57Bl/6 background. Interestingly, the same dose of the same vector generated 20% of normal levels of FIX in a 129 murine background. This difference in levels of FIX detected in identically treated mice of different strains suggests that the genetic background plays an important role in rAAV-mediated FIX transgene expression. Alternatively, the extent of antibodies to canine factor IX may be strain specific. In this study, we tried to determine the effective dose to achieve the therapeutic level of cFIX in a nonrodent large-animal model, the Chapel Hill hemophilia B dog. The first two dogs (C51 and C52) received the same rAAV vector but at a 30-fold lower dose ($3.7–2.8 \times 10^{11}$ particles/kg) than the mice. If the rAAV vector and LSP promoter have similar potencies in dogs as it is in 129 or C57Bl/6 mice, 0.6–2.3% of the normal level of cFIX expression would be expected in these dog. However, the actual cFIX expression level was only ~0.06% of the normal level, 10- to 40-fold less than the expectation. Thus, results obtained by using rAAV-cFIX in rodents did not predict the level of FIX expression we detected in hemophilia B dogs. In contrast, there appeared to be greater concordance between murine and canine models, in both the liver and the muscle (7, 9).

The therapeutic level of cFIX expression was achieved in our third dog (C55) injected with 4.6×10^{12} particles/kg of AAV-LSP-cFIX-WPRE, an AAV vector developed from AAV-LSP-cFIX. The injection of a 12-fold higher dose of the new vector generated a 60-fold greater cFIX expression in C55 than in the lower dose cohort. Importantly, the WBCT was shortened and maintained at 10 min, in the range for normal dogs, and the aPTT was reduced significantly from >140 s (before treatment) to 50 s for the duration of the experiment. These functional data correlated well with the cFIX antigen level, strongly suggesting that the cFIX expressed in the treated dog is responsible for the correction of the hemophilic coagulopathy. Both the increased vector dose and the addition of WPRE likely also helped to achieve the therapeutic level. Besides, C55 had dramatic improvement in its phenotype. It had six bleeding episodes during the 12-month period before vector administration; however, it has not had any bleeds since the injection for over 7 months. D39 was injected with 2.8×10^{12} particles/kg of AAV-LSP-cFIX-WPRE and also exhibited a marked shortening of the WBCT and aPTT. However, it expressed 32 ng/ml of cFIX, lower than we expected based on the data from C55. D39 was a 5-kg puppy and was only 2.8 months old at the time of injection. Whether or not the age and size of the dog play a role in FIX expression level remains to be investigated. Another possible reason is that although C55 and D39 were injected with same batch of vector, D39 was injected 3 months later. Since the vector was stored at 4°C during the intervening 3 months, the titer may have dropped.

Successful expression of sustained therapeutic levels of cFIX in a large animal model of hemophilia B by a single intraportal injection of a rAAV vector shows great promise for success in human clinical trials using AAV-mediated liver-targeted gene therapy to treat hemophilia and other genetic diseases.

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